*J. New York Entomol. Soc.* 99(3):478–486, 1991

# *LIGYROCORIS* (HETEROPTERA: LYGAEIDAE: RHYPAROCHROMINAE) MALE-PRODUCED SCENTS SUGGEST A BIOCHEMICAL CHARACTER SYSTEM FOR SYSTEMATIC ANALYSIS

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*Abstract.*—Gas chromatographic-mass spectral analyses were performed on Tenax®-trapped male specific volatiles (putative pheromones) from sympatrically collected *Ligyrocoris diffusus* (Uhler), *Ligyrocoris sylvestris* (L.) and a group of males intermediate in morphotype, assumed to be hybrids between the two species. Three distinct chemical profiles containing a total of nine insect-derived components were obtained. Two components/peaks were seen in all three chromatograms. Of the remaining seven components, three exhibited by *L. diffusus* were not shown by *L. sylvestris*; similarly four were *L. sylvestris* specific. The volatiles from the presumed hybrid males gave a biochemically hybrid profile, sharing two of the four *L. sylvestris* specific components and all three of the *L. diffusus* specific peaks. Quantitative as well as qualitative differences and commonalities were found among these two *Ligyrocoris* species and the hybrid. These data, in conjunction with preliminary analyses of male volatiles from *Perigenes constrictus* (Say) and *Slaterobius insignis* (Uhler), representatives of two genera both closely related to *Ligyrocoris,* suggest that these male specific scent compounds may be a useful biochemical character system for systematic analysis in the Rhyparochrominae.

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Although there are numerous studies of the so-called "defensive secretions" of both nymphal and adult Heteroptera, evidence of pheromone production in the True Bugs is comparatively scant (Aldrich, 1988). Males commonly emit attractants in the Pentatomoidea (Aldrich et. al., 1984; Harris and Todd, 1980; Hibino, 1985; Knight et. al., 1985; Moriya and Masakazu, 1984; Staddon, 1990; Vrkoc et. al., 1977) and also in the Coreidae (Aldrich et. al., 1982). Ondarza et. al. (1986) studying volatiles collected from *Triatoma mazzottii* Usinger (Heteroptera: Reduviidae) implicated a male attractant produced by females and, in the Miridae, females also seem to be the primary attracting or pheromone producing sex (Graham, 1987; King, 1973).

For the family Lygaeidae, work has focused primarily on the large milkweed bug, *Oncopeltus fasciatus* (Dallas) of the subfamily Lygaeinae. Lener (1969) noted a sweet smell from males of *O. fasciatus,* but no such odor from females. Subsequent analysis revealed that this odor is due to a complex of acetates produced in large quantities by the accessory gland of the metathoracic scent gland, which is markedly reduced in females (Games and Staddon, 1973). They suggested that these male-produced acetates are likely to have a role in the sexual activities of the adults. Aller and Caldwell (1979) revealed that extracts from third instar nymphs and young adult females were attractive to both groups. Older females, however, were not only not attracted to these extracts but also yielded an extract that was repellent to the other two groups.

Outside of the Lygaeinae there is little published information on pheromone pro-

duction. Harrington (1972) suggested a pheromonal role for secretions of the metathoracic scent glands of adults and the abdominal scent glands of nymphs for the blissine *Ischnodemus falicus* (Say). In the large subfamily of mostly ground-dwelling Rhyparochrominae, males of *Perigenes constrictus* (Say) produce a peculiar and highly persistent odor associated with the defecations, which characteristically are smeared in long streaks by males (Sweet, 1964). Sweet further suggested that this odor and smearing behavior might play a part in the species' mating behavior.

Within the myodochine genus *Ligyrocoris,* the "songs" produced by stridulating males of two species are distinct (Thorpe, 1979 MS thesis). Yet, surprisingly, *Ligyrocoris diffusus* (Uhler) males silenced by filling the groves of the stridulitrum with nail polish were just as effective as control males in courting and mating with females (Thorpe and Harrington, 1981). Sometime subsequently, I detected a subtle anise-like odor in culture dishes housing isolated male *L. diffusus.* No such odor was apparent in the dishes housing females. Further behavioral observations revealed that this odor was strongest when a male extruded the pygophore in the process of courting a female; males of *Ligyrocoris sylvestris* (L.) also were noted to produce a distinctive odor (Harrington, unpubl.). With pygophore extrusion, the entire abdomen was elongated, exposing the intersegmental membranes and suggesting an odor source such as the sexually dimorphic ventral abdominal gland opening on the VII-VIIIth intersegmental membrane of males of *Pachylis laticornis* (Heteroptera: Coreidae) (Aldrich et. al., 1982) or the male-specific floral and socket type dermal glands found on abdominal sternite IV of *Dysdercus fasciatus* Signoret (Heteroptera: Pyrrhocoridae) (Lawrence and Staddon, 1975).

If these male produced scents are sex pheromones, they are likely to show species-specificity and, thus, provide biochemical characters for systematic analysis. With this reasoning in mind, the study described below was undertaken to investigate the possible utility of these male scent compounds as a character system.

## DEDICATION

I am pleased to contribute to this festschrift volume honoring James A. Slater. Beginning when I became his graduate student 25 years ago and continuing in collegial dialogues ever since, Jim has taught me an appreciation for the Lygaeidae, the importance of searching for alternative character systems, and the fundamental value of systematics as the basis for all biological science. Even in retirement, he remains active and productive, teaching, as he does best, by presenting an inspiring example to all of us.

## MATERIALS AND METHODS

*Insects. L. diffusus* is a common lygaeid, often found in numbers along roadsides and in weedy fields throughout much of the United States and Canada from NewFoundland to British Columbia (Slater, 1964). *L. sylvestris* is much less commonly encountered, restricted to more mesic habitats, and typically occurs in sparse populations. It is more northern in distribution; Sweet (1964) regards it as a boreal species. In the central sands portion of Wisconsin these two species occur sympatrically in Juneau and Wood counties.

Collections of *Ligyrocoris* made near Babcock, Wood Co., WI and Necedah, Juneau

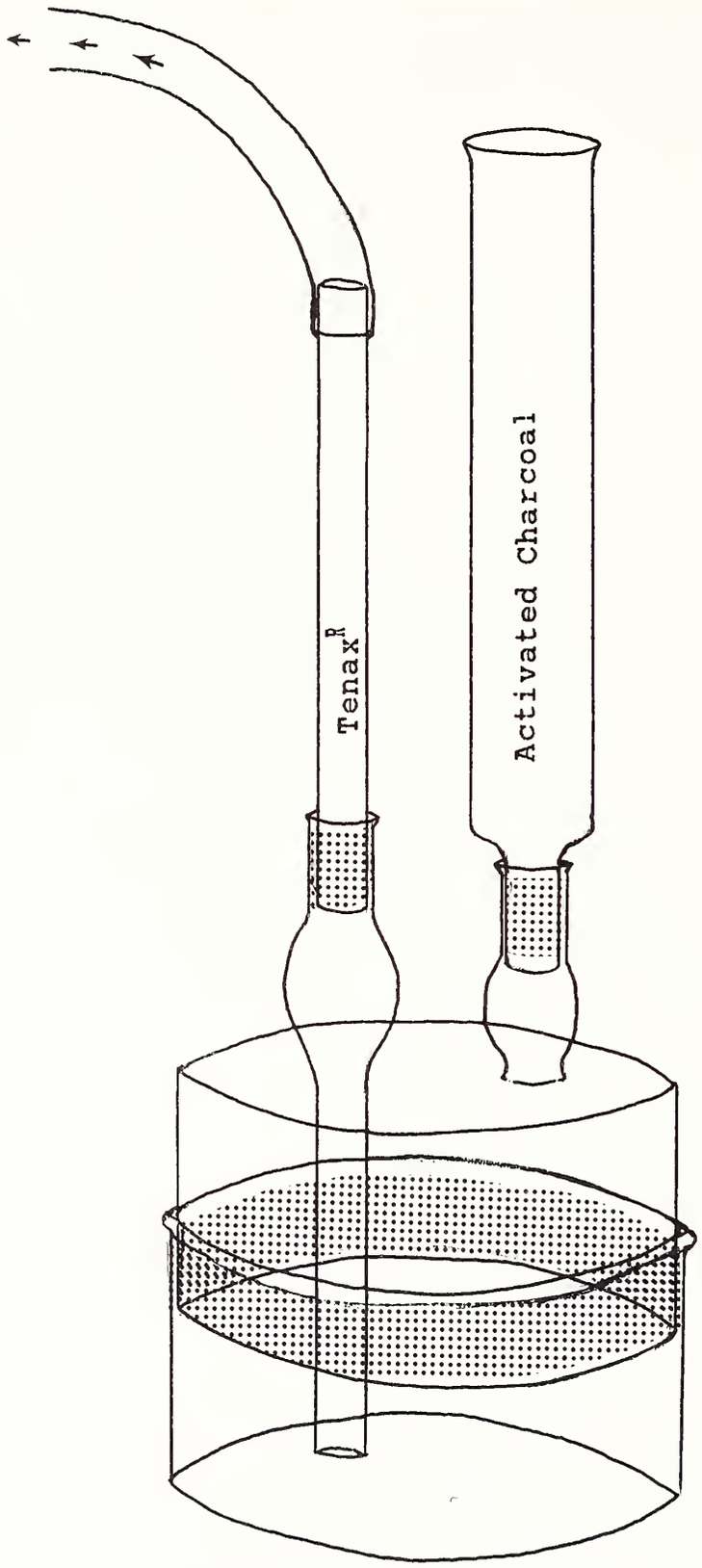


Fig. 1. Modified weighing bottle apparatus for containing insects and collecting volatiles.

Co., WI, in June and July of 1990, included some nymphs which were confusingly intermediate in appearance between the two species. While their coloration, including a distinctive white transverse abdominal band, was more typical of *L. sylvestris,* they had an erect vestiture similar to that of *L. diffusus* but less dense (*L. sylvestris* is virtually devoid of such long upright hairs). When reared to adulthood, these individuals still were not identifiable as either species and represented an intermediate

morphotype; they were tentatively assumed to be hybrids. These presumed hybrids and both *Ligyrocoris* species for this study were provided with water and sunflower seeds, and allowed to mature in the laboratory at 30°C under a 16L:8D photoperiod.

*Chemical analyses.* Males 1–3 weeks old were contained for scent collection in modified 70 × 33 mm weighing bottles fitted with two chimneys ending in ground glass fittings, one for a filter of activated charcoal and the other for a packed absorbent column containing Tenax® (Fig. 1). Insects were confined with sunflower seeds and water, the latter in a 1 dram vial with dental wicking inserted through a hole in the cap and anchored on the floor of the dish with a small piece of PermaPlast® modeling compound. Scent collections were made by pulling a vacuum regulated by a flowmeter at 15 psi through the apparatus described above. Since stored, unused Tenax® readily absorbs contaminants from the air, blank collection dish controls (i.e., with seed and water but without insects) were run for each Tenax® stock used. A similar volatile collection was made from females of *L. diffusus.*

Each collected scent or control sample was eluted by pouring ca. 3–5 ml of 99⁺% capillary GC n-Hexane (Sigma Chemical, St. Louis, MO) through the column and collecting in a 13 × 100 mm culture tube with a Teflon® lined screw cap. Eluted samples were stored in a standard household refrigerator freezer (ca. −15°C) for 1–4 weeks. Prior to analysis, samples were concentrated under nitrogen to ca 30 μl. For gas chromatography-mass spectrometry analysis, a 1 μl portion of each sample was injected into a 10 m × 0.19 mm i.d., DB-5, bonded phase capillary column (J and W Scientific, Folsom, CA) in a Hewlett-Packard 5890A Gas Chromatograph coupled with a Hewlett-Packard 5970 Mass Selective Detector and a Hewlett-Packard 9133 Data System. Analyses were done using temperature programming, with an initial temperature of 70°C, a final temperature of 200°C and a program rate of 10°C/min. Minor variability in injection procedure may produce minute variations in retention times for the same peak in different runs. To compensate for this variation, and for possible different compounds with the same retention times, mass spectrograms were examined to check the identity of peaks with the apparent same retention times among male odor and control samples. Samples analyzed were approximately 4 insect day equivalents (IDE) of scent output each for *L. diffusus* males and females and *L. sylvestris* males and 2 IDE for the presumed hybrid males.

In an effort to localize a possible source of the observed male odors, eight males of *L. diffusus* were killed by freezing and the bodies separated into head, thorax and abdomen portions. Half of the abdomens were further treated by pulling and stretching to expose the pygophores and intersegmental membranes. Four of each type of body part were soaked for 2 min in hexane and the resulting extracts analyzed as described above for the volatile collections.

## RESULTS

Total ion chromatograms for the male odors of *L. sylvestris,* the field-collected presumed hybrids, and *L. diffusus,* are shown in Figure 2A, B, and C, respectively. Comparison of these male odor chromatograms with the Tenax®, seed and water controls revealed that the peaks numbered in Figure 2 are all bug produced compounds. The assemblage of peaks for compounds with retention times greater than 15 min (most readily apparent in Fig. 2A) seem to be minor components found in

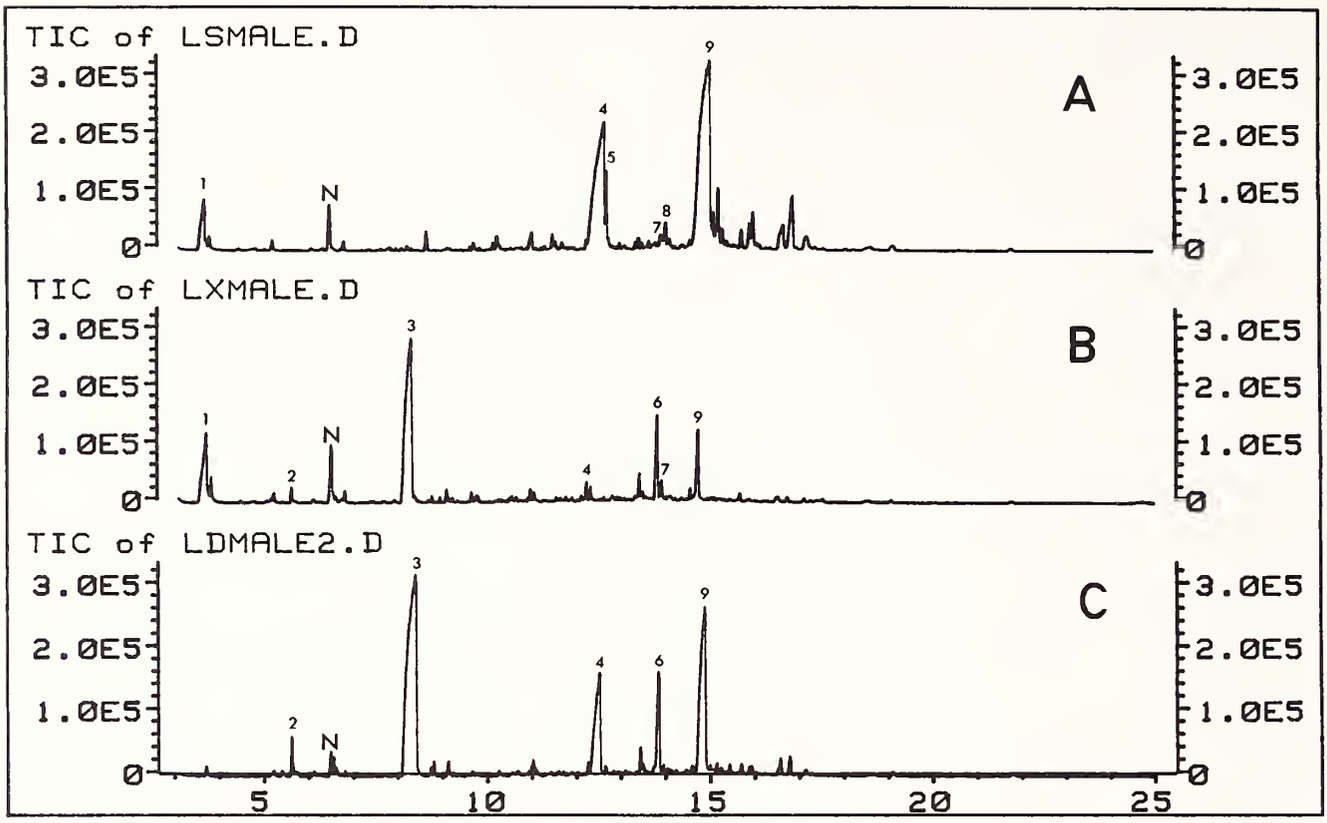


Fig. 2. Total ion chromatograms of male-produced volatiles: A. *Ligyrocoris sylvestris* (L.). B. Presumed hybrids between *L. sylvestris* and *L. diffusus*. C. *Ligyrocoris diffusus*. (Uhler). Numbered peaks represent insect-derived components; N = naphthalene.

all three male group odors; these are not numbered or discussed. The peak labeled "N" (retention time ca. 6.5 min) represents naphthalene, a pervasive and unavoidable contaminant in the atmosphere from the close proximity of the departmental insect collection.

Examination of the profiles in Figure 2 reveals that these male-produced odors involve a complex of compounds. Both species share the compounds represented by peaks 4 and 9, while *L. sylvestris* (Fig. 2A) lacks the compounds represented by peaks 2, 3 and 6 and *L. diffusus* (Fig. 2C) lacks those represented by peaks 1, 5, 7 and 8. Peaks 5 and 8 represent compounds apparently unique to *L. sylvestris,* and not found in the volatiles of either *L. diffusus* or the hybrid males.

Comparison of Figure 2B with the two species' chromatograms reveals that the odors produced by the presumed hybrid males provide a third distinctive chromatogram or profile that is "hybrid" in chemical composition, having some peaks in common with each species but lacking others. The hybrids exhibit peaks 4 and 9 which the two species also have in common. However, it should be noted that the compounds represented by these two peaks are present in much less abundance in the hybrids than in either of the species where they represent major components of the odor complex. In addition, the hybrids share peaks 1 and 7 with *L. sylvestris* and peaks 2, 3 and 6 with *L. diffusus.* The hybrid male profile is closer to that of *L. diffusus* with which it shares peaks 2–4, 6 and 9. Only peaks 1, 4, 7 and 9 are found in common in the complex of compounds exhibited by the hybrids and *L. sylvestris.*

Analysis of the volatiles collected from female *L. diffusus* revealed none of the peaks characterizing the male chromatogram for that species and, in fact, no peaks

occurred in the range of retention times corresponding to the complex of compounds produced by the three male groups studied.

The body part extracts of *L. diffusus* males each showed trace amounts of peaks 3 and 4, with the greatest amount being found, as expected, in the soak of the pulled/stretched abdomens.

## DISCUSSION

The strong scent detected olfactorily when a courting male extrudes his pygophore suggested that the odor might be emitted from the pygophore or some other posterior portion of the abdomen. Indeed, among the body part soak extracts, the pulled/stretched abdomens extract gave the largest peaks. However, even these peaks were minor, indicative of very small amounts of the compounds represented by peaks 3 and 4 only and not the other peaks. This negligible yield (which lacks a number of components when compared to the analyzed volatiles of *L. diffusus*) obtained by soaking of body parts suggests that there may not be reservoirs of the compounds making up the male odors but that, instead, these compounds may be stored as precursors and only synthesized upon release. This might also explain why no volatiles were revealed in gas chromatographic analysis of extracts taken from ventral abdominal glands of males of *O. fasciatus* (Aldrich, 1988) and argues for the desirability of a volatile collection method of obtaining suspected pheromones for bioassay. Another reason that volatile collection should be the method of choice is that, where vapor pressures and functionality of pheromone components differ markedly, the liquid phase composition, which is commonly collected directly for a glandular or body part source, may bear little resemblance to the vapor phase or actual pheromone blend (Brand, 1985). Volatile collections are also much cleaner samples, being uncontaminated with other body materials soluble in the same solvent system, and represent the actually emitted compounds in their natural proportions.

Other gas chromatographic analyses of previous volatile collections from male *L. diffusus,* including adults freshly collected from the field and individuals reared in the laboratory for successive generations, indicate the that profile seen in Figure 2C does not vary intraspecifically (Harrington, unpubl.) and suggest that all compounds represented are synthesized by the insects independent of diet (i.e., wild seed choice in the field did not produce a profile different from the restricted laboratory diet of sunflower seeds).

The male odors of both *Ligyrocoris* species studied are sweet and pleasant, not at all like the distinctive buggy odor often produced when Heteroptera are disturbed, which is typically referred to as a "defensive secretion." Aldrich (1988) cautioned that the herding and containment of Heteroptera can cause release of defensive secretions which confound efforts to analyze other volatiles such as possible pheromones. In my experience this is certainly true of some easily agitated species, including a number of Lygaeidae. *Ligyrocoris* species, however, by comparison are very docile, only releasing the characteristic odor of defensive secretion in extreme circumstances such as restraint and gentle squeezing. Thus, I am relatively confident that the volatiles collected for analysis did not include appreciable alarm or defensive secretions.

Preliminary behavioral studies indicate that *L. sylvestris* females are attracted to conspecific male odor extracts applied to absorbent discs, but, surprisingly, *L.*

*diffusus* females in the same test context are not attracted to male *L. diffusus* odors (J. Fetter, pers. comm.). It is likely that the *L. diffusus* females tested simply were not responsive at the time of assay. However, it is possible that the male scent promotes aggregation in *L. sylvestris,* while some other role(s), such as an aphrodisiac or mate acceptance effect could be envisioned for *L. diffusus.* Further studies are in progress to investigate the influences or roles of male scents, both intra- and intersexually and specifically, in the reproductive behavior of these two species of *Ligyrocoris.* The fact that these scents are produced by the male only suggests a sex pheromone or courtship role, possibly acting as species recognition cues or premating reproductive isolating mechanisms. This supposition is substantiated partially by the species-specificity demonstrated in the current study, although the identification of naturally occurring hybrids raises some question about the efficacy of this chemical communication.

Insect pheromone research naturally has focused on identification, synthesis and deployment of pheromones for control of pest species. Yet, relatively early, it was recognized that, since these compounds or compound blends usually exhibited species specificity, they could be utilized taxonomically for species identification or recognition of cryptic/sibling species (Roelofs and Comeau, 1969). The relative inaccessibility of pheromones, compared to morphological characters, have largely kept these biochemical characters from being used systematically for much other than species identification. The potential for their use in phylogenetic analysis and classification has been underutilized but increasingly sensitive equipment which allows analysis of minute amounts of secretions or volatiles may change this. Also, as isolated studies of the pheromones of a few species continue to accumulate in the literature, these chemical characters will become more available for systematic analyses (Renou et. al., 1988).

In the current study, gas chromatographic-mass spectral analyses of male odors or collected volatiles from two species of *Ligyrocoris* have revealed species specificity in the patterns or profiles presented by the total ion chromatograms and the presumed hybrids gave yet a third distinctive chromatogram. The chromatograms, with verification by mass spectra, clearly show both quantitative/relative abundance and qualitative differences with unique peaks and peaks in common between the species. If those peaks in common are found in other species of *Ligyrocoris* and related genera, as might be anticipated with the heritable biosynthetic pathways suggested by the hybrid data, then the complex of compounds making up these male-produced scents should provide a very good biochemical character system to corroborate or refute systematic analyses based on morphological character systems. Preliminary analyses of male volatiles from *Perigenes constrictus* (Say) and *Slaterobius insignis* (Uhler), two other genera belonging to the same monophyletic lineage as *Ligyrocoris* (Harrington, 1980), show distinct profiles including some peaks in common (possible synapomorphies) suggesting that the male specific volatiles of these bugs have utility for systematics. Further work in pursuing this potential and behavioral studies of the biological role(s) of these male specific scents are in progress.

ACKNOWLEDGMENTS

I thank R. W. Howard, USDA-ARS Grain Marketing Research Laboratory, Manhattan, KS for access to and instruction in the use of the gas chromatograph-mass spectrometer and for

reading a draft of the manuscript. This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin–Madison and by Federal Hatch support, Project no. 3150.

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Received 5 October 1990; accepted 30 January 1991.